



Do *Lasius niger* ants punish low-quality black bean aphid mutualists?

A. Vantaux*, T. Parmentier, J. Billen, T. Wenseleers

Laboratory of Entomology, Zoological Institute, Catholic University of Leuven

ARTICLE INFO

Article history:

Received 23 May 2011

Initial acceptance 1 July 2011

Final acceptance 12 October 2011

Available online 26 November 2011

MS. number: 11-00425

Keywords:

ant
aphid
cooperation
honeydew
melezitose
mutualism

Interspecific mutualisms, that is, reciprocally beneficial relationships between different species, are ubiquitous in nature and ecologically very important. The long-term stability of mutualisms, however, may be threatened by the emergence of 'cheaters' or by the abandoning or extinction of one of the mutualistic partners. Recently, we found large genetic variation in a key trait that helps to maintain the trophic mutualism between ants and aphids, whereby the aphids offer sugary-rich honeydew to the ants, which, in exchange, protect the aphids against natural enemies. In particular, we found that about half of all *Aphis fabae* black bean aphid clones screened do not invest in producing a key honeydew sugar, the trisaccharide melezitose, which also happens to be very attractive to and nutritionally valuable for ants. The aim of the present study was to find out whether ants have any mechanisms available to 'punish' such low-quality melezitose-deficient clones, for example by reducing their visit rate and level of protection against natural enemies. Surprisingly, we found that the visit rates of low-melezitose-secreting clone colonies were no different from those composed of normal, high-melezitose-secreting clones. Nevertheless, our results suggest that ants were more likely to collect honeydew from the high-melezitose-secreting clones. Overall, we conclude that the stability of this particular ant–aphid mutualism is not maintained by active punishment and argue that this may be the reason why the low-melezitose-secreting clones manage to attain such high frequencies in natural populations.

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Interspecific mutualisms, that is, reciprocally beneficial relationships between different species, are ecologically widespread, important at several levels of biological organization and vital for ecosystem functioning (Boucher 1985; Bronstein 1994; Herre et al. 1999). The long-term stability of mutualisms, however, may be threatened by the emergence of 'cheaters', which accept the benefits of the mutualistic partner without providing anything in return, by a shift in the cost-benefit ratio of the interaction, which can cause one species to abandon the mutualism, or, particularly for obligate mutualisms, by the extinction of one of the mutualistic partners (reviewed in Sachs & Simms 2006). Overall, mutualism is favoured when the benefits of the interaction outweigh the costs of investing in cooperative, mutualistic traits (West-Eberhard 1975; Connor 1995; Sachs et al. 2004; Foster & Wenseleers 2006). Such a low cost to benefit ratio is thought to apply for example to the ant–aphid mutualism, in which the aphids offer sugary-rich honeydew to the ants, which, in exchange, protect the aphids against natural enemies (Way 1963; Hölldobler & Wilson 1990; Stadler & Dixon 2005). This is because the benefits of being tended by ants and being better protected against natural enemies are potentially very large, since

predation can quickly drive aphid colonies to extinction (Dixon 1998). In addition, since the aphids feed on plant sap which is of low nutritional value, they have to process large quantities of sap to be able to collect enough nitrogen. Consequently, the honeydew they excrete, and which contains high concentrations of various sugars and also some amino acids, is in part a waste product and can be produced at a relatively low cost (Kennedy & Stroyan 1959; Douglas 2003; Stadler & Dixon 2005, 2008).

At the same time though, it has been shown that increased ant attendance caused by the production of more or better quality honeydew can also be costly (Fischer & Shingleton 2001; Yao & Akimoto 2001, 2002). This is because the ants might force the aphids to excrete at a higher than optimal rate through their behaviour of tapping the aphids with their antennae or via the release of particular chemical stimuli which cause them to release more honeydew (Yao & Akimoto 2001). The consequent increase in excretion rate lowers the amount of nitrogen that can be allocated to growth and reproduction, and results in a metabolic cost caused by the conversion of mono- and disaccharides to tri- and oligosaccharides, which is required to reduce osmotic pressure in the aphids' guts at higher feeding rates (Stadler & Dixon 1998, 2005; Yao & Akimoto 2001, 2002). In line with this interpretation, it has been shown that in the absence of natural predators, ant attendance results in a prolonged developmental time, delayed offspring production, smaller gonads, fewer well-developed embryos and

* Correspondence: A. Vantaux, Laboratory of Entomology, Zoological Institute, Catholic University of Leuven, Naamsestraat 59, Box 2466, B-3000 Leuven, Belgium.
E-mail address: amelie.vantaux@gmail.com (A. Vantaux).

a reduced mean relative growth rate in *Aphis fabae cirsiacanthoidis* (Stadler & Dixon 1998), in reduced colony growth and alate production in *Aphis fabae fabae* (Vantaux 2011) and in the production of smaller and less fecund adults in *Tuberculatus quercicola* (Yao et al. 2000). Furthermore, the fact that ant attendance results in a facultative increase in the production of di- and trisaccharides and amino acids in *T. quercicola* honeydew (Yao & Akimoto 2001, 2002) and in an increase in the production of melezitose in *Chaitophorus populeti* and *Chaitophorus populialbae* (Fischer & Shingleton 2001) suggests that producing high-quality honeydew also entails a metabolic cost. Hence, despite the fact that a low cost to benefit ratio can readily allow the ant–aphid mutualism to evolve, the mutualism is also open to abandonment by one of the partners. For example, the aphids would be selected to leave the mutualism in situations where ant attendance would be too costly, and the ants would be expected to leave the mutualism when they have the opportunity to switch to other aphids that produce better quality honeydew (Stadler et al. 2001; Shingleton & Stern 2003; Stadler & Dixon 2005; Sachs & Simms 2006). Indeed, this may explain why only one-third of the aphid species in Europe are obligate mutualists and another third are only facultative mutualists, with the remainder not being ant-tended (Bristow 1991; Stadler 1997).

Recently, we found that in the facultative ant-tended black bean aphid, *A. fabae*, about half of the aphid clones do not appear to invest in producing a key honeydew sugar, the trisaccharide melezitose, which happens to be highly attractive to ants (Völkl et al. 1999; Fischer et al. 2005; Detrain et al. 2010) and nutritionally very valuable for ants (Boevé & Wäckers 2003), and that these melezitose-deficient clones also produce less total sugar in their honeydew (Vantaux et al. 2011b). We hypothesized that these clones differ in their melezitose secretion based on differences in their carbohydrate metabolism, for example in the sucrose/alpha-transglucosidase activity in their guts (Ashford et al. 2000; Cristofolletti et al. 2003; Price et al. 2007), and that clones with increased sucrose and transglucosidase activity would be able to ingest larger amounts of plant sap and produce larger quantities of melezitose and total sugar. We also found that even though the high-melezitose-secreting clones have a higher mean relative growth rate and a greater competitive ability, the low-melezitose-secreting clones produce relatively more alates, and that this gives them a better ability to disperse and colonize new host plants (Vantaux 2011). Hence, it appears that the two types of clones capitalize on different aspects of their fitness, and that this allows them to coexist in the population. However, given that melezitose is a strong ant attractant (Duckett 1974; Kiss 1981; Völkl et al. 1999; Woodring et al. 2004; Fischer et al. 2005; Detrain et al. 2010) and is nutritionally very valuable for ants (Boevé & Wäckers 2003), and that the high-melezitose-secreting clones also produced more total sugar in their honeydew (Vantaux et al. 2011b), we also expected that ants would prefer to tend the high-melezitose-secreting aphid clones, thereby causing the low-melezitose-secreting ones to be less well protected against natural enemies. Akin to the punishment of uncooperative ‘cheaters’ observed in some mutualisms (West et al. 2002; Kiers et al. 2003; Bshary & Grutter 2005; Marco et al. 2009; Edwards et al. 2010; Jandér & Herre 2010), such a preference of ants to tend high-melezitose, high-sugar-secreting clones could select against aphids producing low-quality, nutrient-poor honeydew.

The aim of the present study was to find out whether ants indeed show a preference to tend high-quality, high-melezitose-secreting clones. It has been shown that the rate of honeydew secretion and the honeydew sugar concentration, particularly that of the trisaccharide melezitose, are key in determining the degree of ant attendance of different aphid species (Völkl et al. 1999; Woodring et al. 2004; Fischer et al. 2005). Typically, this leads to

ant attendance hierarchies, in which the ants preferentially visit the aphid species that produce the best quality honeydew (Addicott 1978; Völkl et al. 1999; Fischer et al. 2001), even occasionally preying upon the ones that produce the least amount of honeydew (Sakata 1994, 1995; Offenberg 2001; Mooney & Tillberg 2005). Based on these findings, we expected that if the ants displayed similar preferences at the intraspecific level, the low-melezitose-secreting clones should experience a reduced ant attendance, or increased ant predation, and a reduced protection against natural enemies. In the present study, we tested this prediction using binary choice experiments. Furthermore, given that we found natural *A. fabae* colonies are frequently composed of a mix of up to four different clones (Vantaux et al. 2011a), we tested whether clonal mixing would make it harder for ants to punish low-melezitose-secreting cheaters.

METHODS

Study Organisms

Aphis fabae is a facultative myrmecophilous aphid species which is often tended by the black garden ant, *Lasius niger* (Banks 1958), as well as by various other ant species, depending on which species happens to be locally most abundant. Five distinct *A. fabae* clones were used in this study of which two were collected from bean plants, *Vicia faba*, in the surroundings of Leuven, Belgium, two came from the Agrocampus in Rennes, France, and one was provided by the Rothamsted Research Institute (Hertfordshire, U.K.). To make sure that all these were genetically distinct clones they were first genotyped at seven microsatellite loci following the methods described in Vantaux et al. (2011a). Furthermore, we established using HPLC analysis that two of these were high-melezitose-secreting clones, whereas three were low-melezitose-secreting clones (Vantaux et al. 2011b). Both sets of clones differed about 100-fold in their average melezitose production, as well as about 1.5 times in their total sugar production ($48.98 \pm 6.30 \mu\text{g}/\mu\text{l}$ versus $30.47 \pm 5.13 \mu\text{g}/\mu\text{l}$). For the high-melezitose-secreting clones melezitose represented the dominant honeydew sugar, comprising 57.6% of all the sugars produced, whereas for the low-melezitose-secreting clones, melezitose made up only 0.87% of the sugars produced (Vantaux et al. 2011b). Across 10 clones derived from the same population in Belgium, ca. 45.5% were high-melezitose-secreting clones and 54.5% were low-melezitose-secreting clones, which means that both types coexist stably within the same population (Vantaux et al. 2011b). Of all five clones, we maintained parthenogenetic lineages in the laboratory on broad bean, one of its secondary host plants (Stroyan 1984). Broad beans were grown in potting soil at $20 \pm 1^\circ\text{C}$ under a 16:8 h light:dark photoperiod, which was the same as that used for breeding the aphids. Seeds were provided by Somers Seeds NV, Mechelen, Belgium.

Lasius niger is known to tend various aphid species, including *A. fabae*. Colonies were started from queens collected just after the nuptial flight or were ordered from Antstore, Berlin, Germany. All colonies contained a queen, brood and 50–100 workers. They were kept in glass test tubes which were wrapped with black plastic and filled approximately one-third with water, with a tightly packed cotton ball placed on top of the water, to reproduce a natural nest environment. The open test tube was placed in a plastic box to provide the ants with a foraging arena ($19.5 \times 9.5 \text{ cm}$ and 5.5 cm deep) of which the sides were coated with Fluon GP-1 to prevent the ants from escaping. The experiments were conducted between September 2009 and February 2011. Ant colonies were fed ad libitum with a honey solution and mealworms, *Tenebrio molitor*, three times a week. Ants were kept under identical temperature and light conditions as used to breed the aphids and bean plants.

Binary Choice Experimental Set-up 1

Ant colonies were given a choice between a high-melezitose-secreting clone colony and a low-melezitose-secreting clone colony using a Y-shaped bridge. To ensure that approximately equal numbers of aphids were introduced onto each plant, bean plants were grown to a size of 4–5 cm before being infested with 10 adult apterous aphids. Plants and aphids were then allowed to develop for 5 days. Before each trial, the ant colonies were starved for 5 days by replacing the honey solution with water only. The day of the experiment, the plant pots were wrapped in cling film to prevent ants from moving in. In addition, ants and aphids were prevented from escaping by placing the plants in water-filled boxes. Subsequently, the foraging arena and the two aphid-infested plants were connected via a paper bridge (0.5 cm width) and the total amount of ant traffic (ants which were either leaving or going back to their nest) on either side of the Y-bridge was measured once per hour for 20 min for a total of 8 h. During these observations, we also recorded the feeding status of all ants, that is, whether they had a normal-sized gaster or a distended gaster because of honeydew consumption. A distended gaster was recognizable because the tergites are then forced apart by the filled crop. Since we had two high- and three low-melezitose-secreting clones, six different choice combinations were made (each high-melezitose-secreting clone paired up with each low-melezitose-secreting one). We ran five replicates per combination and thus 30 binary choice trials were analysed in total.

Binary Choice Experimental Set-up 2

In a second set-up, ant colonies were given a choice between a high-melezitose-secreting clone colony and a mixed colony consisting of an equal mix of high- and low-melezitose-secreting clones (five aphids of each type were placed on the plant). Before each trial, the ants were starved by supplying them with water only. Plants and aphids were allowed to develop for 2 days before the day of the trial, and we used the same set-up and observation method as described before. Having two high-melezitose-secreting clones and three low-melezitose-secreting clones allowed us to have 12 different choice combinations, in which each of the two high-melezitose-secreting clones were paired up with each of the six mixed colonies (H1–L1, H1–L2, H1–L3, H2–L1, H2–L2, H2–L3). We ran four replicates per combination and thus analysed 48 binary choice trials in total.

Statistical Analyses

From the data, we calculated the visit rate and the honeydew collection ratio. The visit rate was calculated as the average number of ants that passed per hour on each side of the Y-bridge over the total observation period of 160 min collected over a period of 8 h. The honeydew collection ratio was calculated as the proportion of all ants that left the plant and that had a distended gaster. Assuming that the rate of honeydew secretion of high- and low-melezitose-secreting clones is roughly equal, the latter ratio measures the probability that ants would collect honeydew from each type of clone. To satisfy normality assumptions, visit rate and honeydew collection ratios were log and arcsine-square-root transformed, respectively. Subsequently, the log-transformed visit rate and the arcsine-square-root-transformed honeydew collection ratio of the high- and low-melezitose-secreting clone colonies (or high- and mixed-clone colonies) were compared using a general linear model (GLM), whereby the level of melezitose secretion (high or low) as well as the two aphid colonies that were used in each trial were coded as fixed factors and colony was nested within melezitose. All

tests performed were two tailed and averaged values are reported as mean \pm SE. All analyses were conducted with Statistica version 9 (Statsoft, Tulsa, OK, U.S.A.).

RESULTS

Binary Choice Experimental Set-up 1

The visit rates of the high-melezitose-secreting clone colonies (31.58 ± 5.95 ants/h) and the low-melezitose-secreting clone colonies (26.97 ± 3.66 ants/h) were not significantly different from one another ($F_{1, 55} = 0.069$, $P = 0.79$; Fig. 1), and the different high- or low-melezitose-secreting aphid clones did not have a significant difference in their visit rates either ($F_{3, 55} = 0.473$, $P = 0.70$). Predation of the ants on the aphids was so low that it did not affect aphid population dynamics. In fact, over all 30 trials and a total observation of 80 h, only two ants were observed retrieving an aphid back to the colony. In both of these cases the aphid that was preyed upon was a low-melezitose-secreting one and no significant bias towards low-melezitose aphid clones was detectable (two-tailed sign test: $P = 0.5$). Hence, there was no evidence that the ants punished the low-melezitose-secreting cheater clones via modulation of either their visit rate or predation rate. The honeydew collection ratio, however, was slightly higher for the high-melezitose-secreting clone colonies (0.84 ± 0.02) than for the low-melezitose-secreting ones (0.68 ± 0.03 ; $F_{1, 55} = 13.207$, $P = 0.0006$; Fig. 2), and the honeydew collection ratio was also significantly different for the high- and low-melezitose-secreting clones ($F_{3, 55} = 2.792$, $P = 0.048$). Fisher least significant difference post hoc tests showed that H2 had a significantly higher honeydew collection rate than all other clones.

Binary Choice Experimental Set-up 2

The visit rates of the high-melezitose-secreting clone colonies (9.26 ± 0.91 ants/h) were no different from those of the high + low-melezitose mixed clone colonies (10.51 ± 0.90 ants/h; $F_{1, 88} = 0.4225$, $P = 0.63$; Fig. 3) and there were no significant differences in the visit rate of the different high-melezitose and mixed-clone aphid colonies used ($F_{6, 88} = 1.4543$, $P = 0.20$). As in the first experiment, predation of the ants on the aphids was so low that

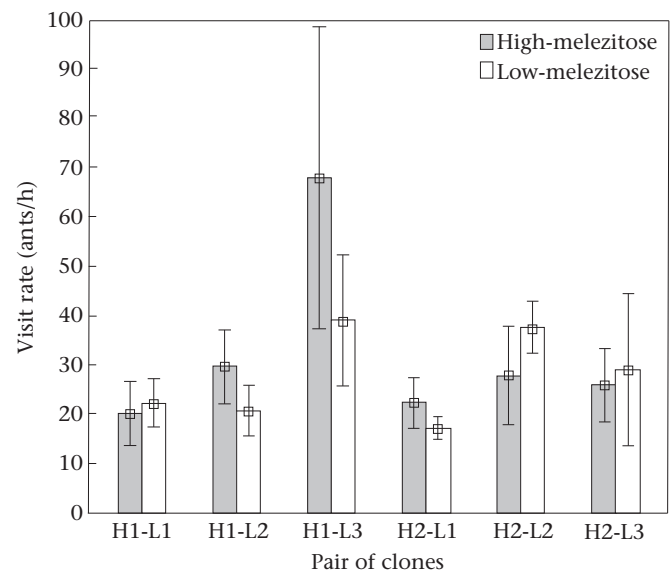


Figure 1. Visit rates (ants/h) of *L. niger* to high- and low-melezitose-secreting *A. fabae* colonies. Means are shown \pm SE.

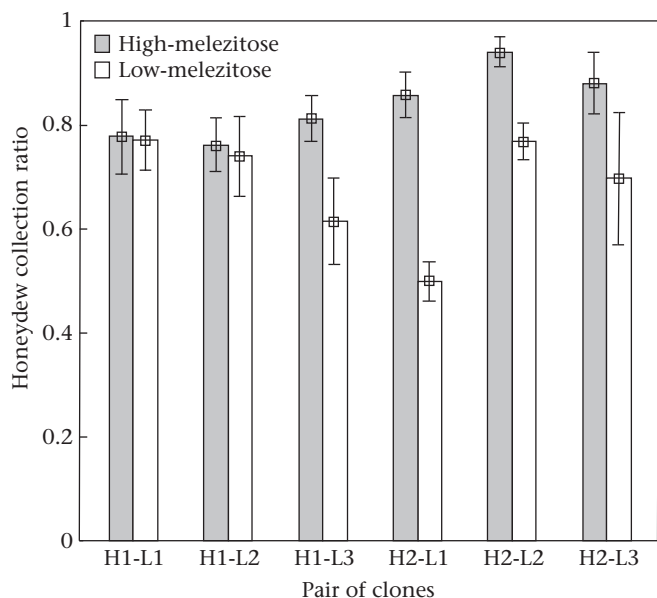


Figure 2. Honeydew collection ratios of *L. niger* returning from high- and low-melezitose-secreting *A. fabae* colonies. Means are shown \pm SE.

it would not significantly affect aphid population dynamics: over all 48 trials and a total observation of 128 h, only three ants were observed retrieving an aphid back to the colony. In two of these cases the aphid that was preyed upon came from the high/low melezitose mixed-clone colony rather than the high-melezitose-secreting one, although again no significant bias towards low-melezitose aphid clones was detectable (two-tailed sign test: $P = 1$). Hence, there was no evidence that the ants were able to punish the low-melezitose-secreting clones when they occurred in a mix with high-melezitose-secreting ones via modulation of either their visit rate or predation rate. In addition, in this experiment, the

honeydew collection ratios were not significantly different between the high-melezitose-secreting clone colonies (0.62 ± 0.04) and the high- and low-melezitose mixed-clone colonies (0.57 ± 0.04 ; $F_{1, 88} = 0.9191$, $P = 0.34$; Fig. 4), and there were no significant differences in the honeydew collection rate of the different aphid high-melezitose and mixed-clone colonies used ($F_{6, 88} = 0.1349$, $P = 0.43$).

DISCUSSION

Since the rates of honeydew secretion, the total concentration of sugars and the presence of melezitose are the main factors determining the degree of ant attendance of different aphid species (Völkl et al. 1999; Woodring et al. 2004; Fischer et al. 2005), we were expecting that when ants had a choice between high- and low-melezitose-secreting clones, they would also visit the latter less. A decreased visit rate in turn would imply that, in nature, the low-melezitose-secreting clones would not be as well protected by ants against natural enemies, so that the low-melezitose-secreting clones would end up being punished relative to more cooperative aphid clones that produced better quality, more nutrient-rich honeydew. Surprisingly, however, the results from our binary choice experiments show that the low-melezitose- and low-sugar-secreting clones did not suffer reduced ant visit rates compared to normal high-melezitose-, high-sugar-secreting ones, either when they occurred as pure clone colonies or when they occurred in a mix with high-melezitose-secreting clones. This means that, in nature, the low-melezitose-secreting clones would still benefit from the ants' protective services even though they invest to a lesser extent in the production of high-quality, nutrient-rich honeydew. The visit rate and honeydew collection ratio were slightly lower in the mixed-clone experiment. This might be because the aphid colonies were left to grow for 5 days in the first experiment versus for only 2 days in the second experiment, resulting in slightly smaller aphid colony sizes in the second experiment. These small aphid colonies were probably not excreting enough honeydew to satisfy the desired food volume of

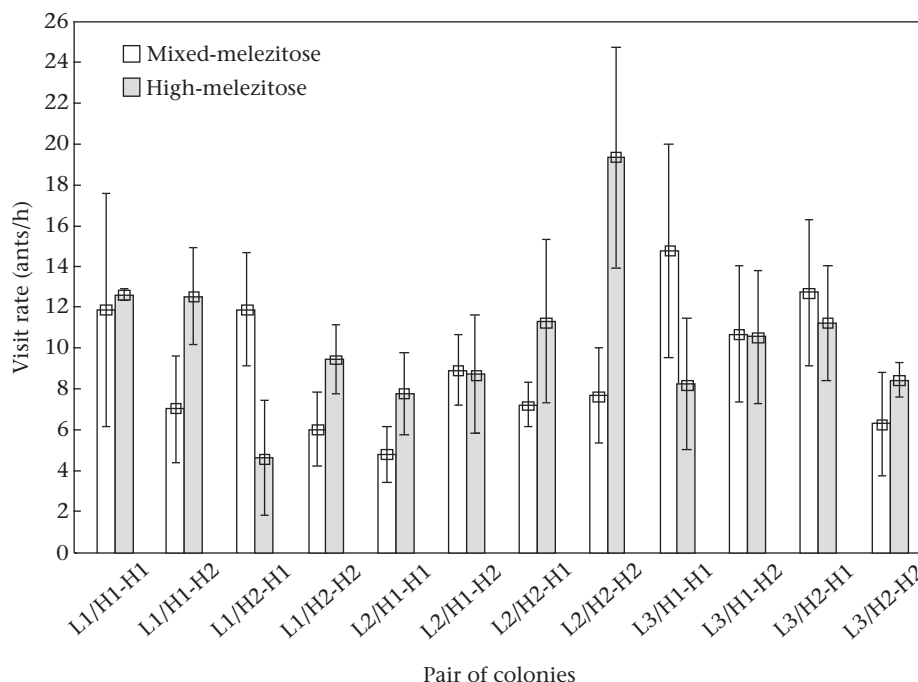


Figure 3. Visit rates (ants/h) of *L. niger* to high-melezitose-secreting *A. fabae* colonies and high- and low-melezitose mixed-clone colonies. Means are shown \pm SE.

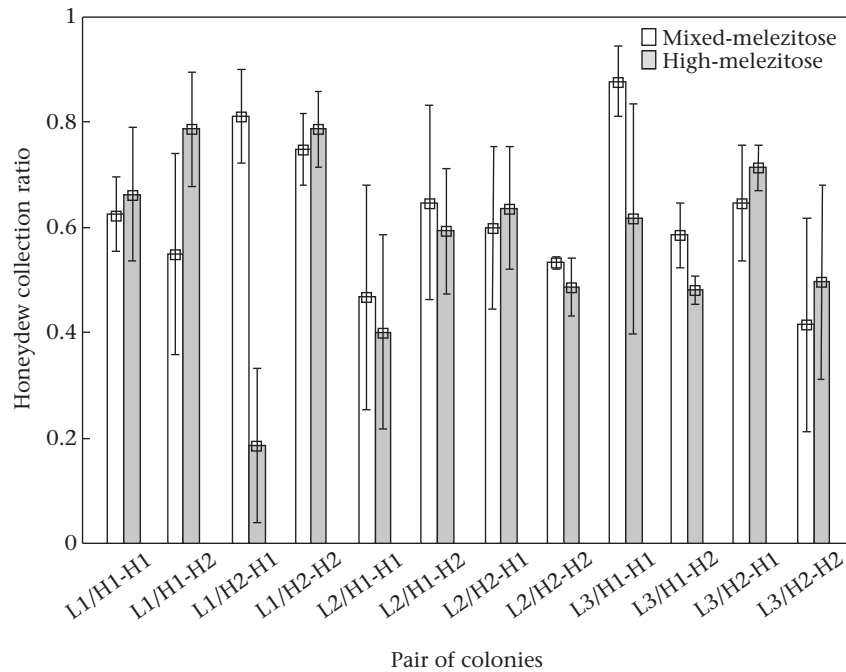


Figure 4. Honeydew collection ratios of *L. niger* returning from high-melezitose-secreting *A. fabae* colonies and high- and low-melezitose mixed-clone colonies. Means are shown \pm SE.

workers and trigger trail-laying behaviour (Mailleux et al. 2000, 2003b). The aphid colony sizes used, however, were well within the range of the naturally observed colony sizes (Vantaux et al. 2011a). Indeed, this might explain why *A. fabae* is only a facultative mutualist not always relying on ants, as it might not be productive enough to stimulate ant attendance on many occasions. Furthermore, even if ant colonies with brood exhibit a higher mobilization of foragers (Portha et al. 2002), in small ant colonies only half of the ants showed trail-laying behaviour (Mailleux et al. 2003a) and thus collective selection of the food source might be rather limited.

Our results also showed that the ants only very rarely preyed on the aphids, and that any predation that did occur was not selectively targeted towards low-melezitose-secreting clones. These results contrast with those of Sakata (1994, 1995), who showed that when *L. niger* is provided with a choice of different aphid species producing different amounts of honeydew, the ants tend to prey on the species that produce the least amount of honeydew. Similarly, Offenberg (2001) observed 1.9% of predation by *L. niger* ants provided with *A. fabae* colonies and an increase to 12.1% when concurrently fed with honey solution. Possibly, the differences between the results of Offenberg (2001) and ours could be because of the different lengths of the starvation periods used in the two experiments, the use of differently sized aphid populations (15 aphid-infested plants in Offenberg's experiments and a higher aphid density per ant leads to increased predation in *L. niger*, Sakata 1994) or the fact that the ants find it easier to select among different aphid species than among different clones of the same species. The latter hypothesis seems unlikely though, given that the ants provided with bigger aphid colonies in the first experiment seemed to prefer to collect honeydew from the high-melezitose-secreting clone colonies. Hence, the ants did seem to be able to be selective about which honeydew they brought back to the nest. The fact that the ants did not display active partner choice at the level of whole aphid colonies, but that they were selective in terms of which honeydew they collected from individual aphids might be explained by the fact that in an earlier genetic study of

A. f. cirsiacanthoidis and *A. f. fabae* we found that 32% and 67% of all naturally occurring aphid colonies were polyclonal, consisting of a mix of up to four different clones (Vantaux et al. 2011a). Hence, even if low-melezitose-secreting clones co-occurred in a mix together with high-melezitose-secreting ones, discrimination at the level of the individual aphids would still allow the ants to collect only high-quality honeydew. Indeed, *L. niger* workers can recognize whether an aphid has been tended by other ants and whether or not honeydew was provided (Sakata 1994). Nevertheless, despite this selectivity in the honeydew that the ants collected, the visit rates and predation rates of the ants were not different between high- and low-melezitose-secreting clones. This implies that the ant–aphid mutualism is not maintained by active partner choice or punishment. Probably, this explains why the low-melezitose-secreting clones manage to attain such high frequencies in natural populations (ca. 50% of all clones surveyed in Belgian populations of *A. fabae*, Vantaux et al. 2011b). Detailed measurements of the fitness of the high- and low-melezitose-secreting clones in the field would shed more light on how the two types of clones manage to be stably maintained in the population.

Acknowledgments

This project was supported by the FWO-Flanders (project N° G.0699.08) and a Program Financing grant ('Eco- and Socio-Evolutionary Dynamics', project N° PF/10/007) of the KULeuven Research Fund.

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